ELSEVIER

Journal of Chromatography A, 853 (1999) 321–335

JOURNAL OF CHROMATOGRAPHY A

Analysis of leptin gene expression in chickens using reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection

Mark P. Richards*, Christopher M. Ashwell, John P. McMurtry

US Department of Agriculture, Agricultural Research Service, Livestock and Poultry Sciences Institute, Growth Biology Laboratory, Beltsville, MD 20705-2350, USA

Abstract

Leptin is a peptide hormone product of the obese (*ob*) gene that functions in the regulation of appetite, energy expenditure and reproduction in animals and humans. We have developed a technique using capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) for the analysis of chicken leptin (261 base pairs, bp) and β-actin (612 bp) double-stranded DNA products from reverse transcription polymerase chain reaction (RT-PCR) assays. Amplicons were separated using a DB-1 coated capillary (27 cm×100 μm I.D.) at a field strength of 300 V/cm in a replaceable sieving matrix consisting of 0.5% hydroxypropylmethylcellulose (HPMC) in 1X TBE (89 m*M* Tris–base, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.3) buffer with 0.5 μg/ml EnhanCE fluorescent intercalating dye. RT-PCR samples (1–2 μl) were diluted 1:100 with deionized water and introduced into the capillary by electrokinetic injection. Separations were completed in less than 6 min and the total time required per sample, including capillary conditioning, was 8 min. We have applied RT-PCR–CE–LIF to determine the effects of insulin and estrogen treatment on leptin gene expression relative to that of β-actin in chicken liver and adipose tissue. In addition, we have constructed a chicken leptin mRNA competitor (234 bp amplicon) and evaluated it for use as an internal standard in the development of a quantitative-competitive RT-PCR assay. Our findings represent the first reported application of capillary electrophoresis to the analysis of leptin gene expression by RT-PCR. Published by Elsevier Science B.V.

Keywords: Leptin; Peptides; Hormones; Actin; DNA; RNA

1. Introduction

Leptin, a polypeptide hormone, whose gene was originally identified by positional cloning techniques in rodents and humans, has been shown to function in the regulation of feeding behavior, energy balance and reproduction [1–4]. Encoded by the obese (*ob*) gene, leptin is synthesized as a 167-amino-acid precursor that is subsequently cleaved to yield the 146-amino-acid (M_r 16 000) mature form of the protein [5]. Mature leptin contains a single, internal disulfide bond and is secreted from its site of synthesis into the plasma. In mammals, leptin is expressed primarily in adipose tissue in direct proportion to the size of energy (fat) stores; although, its expression has also been detected at lower levels in gastric epithelium and in the placenta [6]. Leptin acts through its specific receptor (ObR), a member of the type I cytokine receptor superfamily, which is expressed in a variety of tissues throughout the body

*Corresponding author. Tel.: +1-301-504-8892; fax: +1-301-504-8623.

E-mail address: richards@lpsi.barc.usda.gov (M.P. Richards)

0021-9673/99/$ – see front matter Published by Elsevier Science B.V.
PII: S0021-9673(99)00576-2

[7]. As an afferent signal (lipostat) in a postulated negative-feedback loop between adipose tissue and the central nervous system, leptin binds to its receptors located primarily in hypothalamic sites within the brain activating specific neural pathways that, in turn, modulate feeding behavior and energy balance to maintain energy stores at a set level [8].

The regulation of leptin and leptin receptor gene expression is actively being studied in a number of animal species and humans because recessive mutations in either of these two genes result in the development of the pathological conditions of morbid obesity, diabetes, and infertility [6]. There is also a great deal of interest in understanding what role(s) leptin might play in domestic animal species since feeding behavior, energy balance and reproduction each have a significant impact on the success and/or profitability of animal agriculture [9]. Recently, leptin genes have been identified in both chickens and turkeys and their complete coding regions cloned and sequenced ([10,11] GenBank Accession Nos. AF012727, AF082500, AF082501). Unlike mammals, leptin is expressed in the liver as well as in the adipose tissue of chickens, and this situation, apparently unique to avian species, undoubtedly reflects a prominent role for the liver in the regulation of lipogenesis and energy metabolism [12].

A number of methods have been developed to follow changes in leptin gene expression both at the protein and nucleic acid levels. Leptin protein has been detected and/or quantified in a number of species using a variety of species-specific immunoassay techniques including: radioimmunoassay, enzyme-linked immunosorbent assay, Western blot analysis and immunohistochemical localization [13]. Activity of recombinant leptin has been determined in vivo by subcutaneous, intravenous or intracerebral ventricular injections of the purified recombinant protein with subsequent monitoring of its effects on cumulative feed intake [6]. Leptin functionality (as determined by it receptor binding activity) has also been quantitatively assessed in vitro using a genetically engineered cell line co-transfected with a leptin receptor isoform (ObRb) and a STAT-inducible promoter regulating firefly luciferase [13].

Assays involving reverse transcription and polymerase chain reaction (RT-PCR) are widely used to study leptin gene transcription, however, only a limited number of quantitative-competitive RT-PCR (QC-RT-PCR) assays have been developed to more accurately assess leptin mRNA levels in human adipose tissue and cultured mouse adipocytes [14,15]. A number of investigators have reported on the utility of capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) for semiquantitative and quantitative-competitive analyses of double-stranded (ds) DNA products from PCR and RT-PCR reactions [16–23].

Although, there are no established analytical methods for detecting avian leptin protein, two techniques involving RT-PCR have recently been reported for the analysis of chicken leptin mRNA [10,11]. Thus, RT-PCR assays represent the best currently available method to study leptin gene expression in chickens. The objective of this study was to develop a CE–LIF-based assay for the analysis of chicken leptin dsDNA products from RT-PCR using total RNA isolated from different tissues. Our goal is to be able to rapidly identify and quantify leptin amplicons in RT-PCR reactions using CE–LIF so that an accurate assessment can be made of leptin gene expression in tissues obtained from chickens under different physiological conditions (i.e., fasted, fed, etc.) or at various stages of development and from birds subjected to a variety of experimental treatments.

2. Experimental

2.1. Materials

Hydroxypropylmethylcellulose (HPMC, catalog No. H-7509), estradiol 17-β (catalog No. E-8875) and porcine insulin (catalog No. I-5523) were obtained from Sigma (St. Louis, MO, USA). LIFluor (EnhanCE, catalog No. 477409) and ΦX174 DNA, *Hae*III digest (catalog No. 477414) were from Beckman Coulter (Fullerton, CA, USA). YO-PRO-1 (catalog No. Y-3603) and Sybr Green I (catalog No. S-7585) were purchased from Molecular Probes (Eugene, OR, USA). All other chemicals used in the preparation of buffers were analytical grade or better.

2.2. Animals and experimental treatments

All animal studies were conducted in accordance

with research protocols approved by the Beltsville Animal Care and Use Committee. Day old broiler chicks (Shaver Poultry Breeding Farms, Cambridge, Canada) were raised in brooder batteries until three weeks of age, at which time they were transferred to individual cages for at least an additional week prior to initiating experimental treatments. Four-week-old male birds were injected im with vehicle (propylene glycol) or 20 mg of estradiol 17-β dissolved in vehicle on days one and three. In a second experiment, 12-week-old male birds were injected im with saline or 200 μg porcine insulin/kg body mass/day for a period of four days. Control birds were injected with vehicle only on the designated treatment days. On day five, birds from both experiments were sacrificed and samples of liver and adipose (abdominal fat pad) tissue were obtained and immediately frozen in liquid nitrogen. Tissues were stored frozen at −80°C prior to RNA isolation.

2.3. RT-PCR assay for leptin

Total RNA was isolated from tissue samples using the Tri-Reagent procedure (Life Technologies, Gaithersburg, MD, USA). Isolated RNA was quantified using a UV spectrophotometer (Genequant II, Pharmacia Biotech, Piscataway, NJ, USA). RT reactions (50 μl) consisted of: 5 μg total RNA, 100 units MMLV reverse transcriptase (Promega, Madison, WI, USA), 20 units RNAsin (Promega), 5.5 m*M* dNTPs, and 10 pmol oligo$(dT)_{18}$ primer. PCR reactions were performed in a separate 50 μl reaction containing: 15 m*M* Tris–HCl, pH 8.4, 50 m*M* KCl, 1.7 m*M* $MgCl_2$, 0.1% Triton X-100, 5 μl of the RT reaction, 1.25 units of Taq polymerase (Promega), 1.0 m*M* dNTPs, and 10 pmol each of chicken leptin and β-actin gene specific primers [11]. RT-PCR was performed using a commercial thermal cycler (RoboCycler, Stratagene, La Jolla, CA, USA). PCR thermal cycling parameters were as follows: 1 cycle 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 8 min. The products of RT-PCR were 261 base pairs (bp) (57.5% GC, T_m= 86°C) and 612 bp (50.5% GC, T_m=85°C) for the leptin and β-actin dsDNA amplicons, respectively.

2.4. Primers and leptin internal competitor design

Forward (sense, 5′-CGTCGGTATCCGCCAAG-CAGAGGG) and reverse (anti-sense, 5′-CCAG-GACGCCATCCAGGCTCTCTGGC) primers were designed for chicken leptin as described previously [11]. These primers correspond to bases 134–157 and 369–394, respectively, in the coding sequence reported for chicken leptin (GenBank Accession No. AF082500) and they produced an amplicon of 261 bp (Fig. 1). As an internal standard, a primer set specific for the chicken β-actin gene, was also included in each PCR reaction for the relative

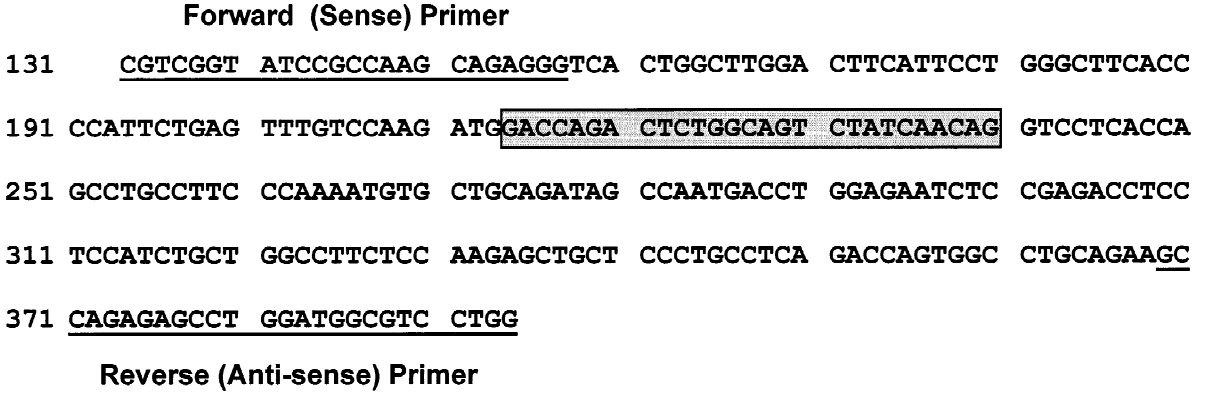

Fig. 1. Sequence of the chicken leptin amplicon (261 bp) showing the positions (underlined) of the forward (sense) and reverse (anti-sense) primers. The numbers to the left reference base pair numbers for the complete coding region of chicken leptin as reported previously (GenBank Accession Nos. AF012727, AF082500). The shaded box highlights that portion of the sequence (27 bp) deleted to construct a chicken leptin internal competitor (see Fig. 2).

quantitation of leptin expression [11]. The chicken β-actin primer set consisted of a forward (sense, 5′-CAAGGAGAAGCTGTGCTACGTGC) and a reverse (anti-sense, 5′-TTAATCCTGAGTCAAGCGCC) pair, which, together, produced an amplicon of 612 bp.

An RNA internal competitor that can be primed for cDNA synthesis and PCR amplification (234 bp amplicon, 58% GC, T_m=86°C) using the same primer set designed to produce the leptin target amplicon (261 bp) was constructed from a plasmid containing a full length leptin coding sequence clone [11]. This plasmid can be used to transcribe either sense or anti-sense RNAs corresponding to the complete coding sequence for chicken leptin (Fig. 2). The coding region (509 bp) was excised from the plasmid by *Eco*RI digestion and isolated by agarose slab gel electrophoresis. The recovered fragment,

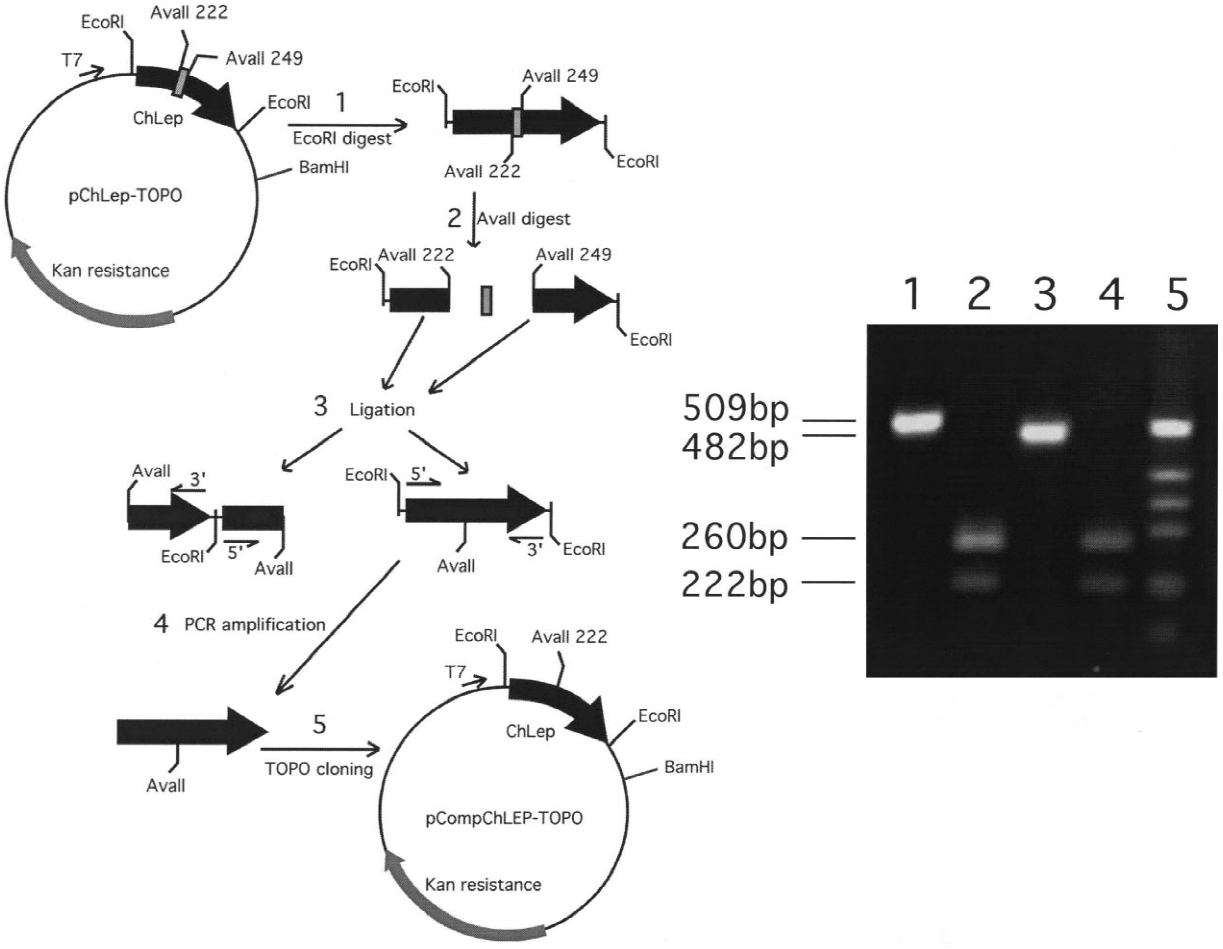

Fig. 2. Procedure used for the construction of a chicken leptin internal competitor by excision (*Ava*II digest) of a 27 bp segment from a DNA fragment (509 bp) containing the full length coding region for chicken leptin. To generate the construct, TOPO cloning, restriction enzyme digestion, ligation of the resulting restriction fragments, and subsequent PCR amplification were employed (left). Verification of this process was achieved by agarose gel electrophoresis with ethidium bromide staining of the restriction fragments corresponding to the leptin full length sequence, internal competitor, and intermediate products (right). Lanes: 1=isolated DNA fragment (509 bp) from *Eco*RI digestion of pClep-TOPO plasmid; 2=products (260 and 222 bp) resulting from the digestion of the 509 bp DNA fragment with *Ava*II; 3=PCR amplified product (482 bp) of the ligated 260 and 222 bp fragments; 4=digestion of the PCR amplified product (482 bp) with *Ava*II resulting in two DNA fragments of 260 and 222 bp in size; 5=1 kb DNA ladder (Life Technologies, Gaithersburg, MD, USA).

excised from the agarose gel, was then digested with the restriction enzyme *Ava*II, which produced three fragments 260, 222 and 27 bp in size. The 260 and 222 bp fragments were isolated by agarose slab gel electrophoresis and ligated using T4 DNA ligase. The resulting ligation product was amplified by PCR using primers corresponding to the 5′ and 3′ ends of the leptin coding sequence (sense, 5′-ATGGAATTCGCCTTCCATATGCCG; anti-sense, 5′-TCAGCATTCCGGGCTAATATCC) and subjected to TOPO cloning (Invitrogen, Carlsbad, CA, USA). The orientation of the coding sequence and the 27 bp deletion were confirmed by automated fluorescent DNA sequencing (ABI 377, Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The deletion construct was also designed to continue the open reading frame and thus delete the codons for nine amino acids corresponding to amino acids 71–80 in the leptin protein sequence.

Competitor and full length leptin synthetic RNAs were transcribed from *Bam*HI-digested plasmids (see Fig. 2) using T7 RNA polymerase and a MAXIscript kit (Ambion, Austin, TX, USA). RNA produced from these reactions was quantified in multiple dilutions using a UV spectrophotometer (Genequant II, Pharmacia Biotech). The synthetic RNAs were diluted to concentrations of 250, 50, 25, 5, and 2.5 amol/μl in nuclease free water. Competitive RT-PCR samples (50 μl) included 5 μl of synthetic competitor RNA, random hexamer primers (instead of oligodT) and for experimental reactions, 5 μg of total RNA. Additional RT reaction components and conditions were described previously and a separate reaction was performed for each of the five dilutions of synthetic competitor RNA. PCR amplifications were carried out using only the leptin gene specific primers as described previously. Control RT-PCR amplifications containing only competitor and full length synthetic RNAs (250 amol/μl) were conducted for verification of equivalent amplification efficiencies.

2.5. CE–LIF

Aliquots (1–2 μl) of RT-PCR samples were diluted 1:100 with deionized water prior to analysis by CE–LIF unless specified otherwise. A P/ACE 5510 (Beckman Coulter) equipped with an argon ion LIF detector and controlled by P/ACE Station software (ver. 1.0) was used for the analysis of all RT-PCR samples. Excitation of the intercalating dyes used in this study was achieved at 488 nm and emission was detected at 520 nm. The CE instrument was configured for reversed polarity (i.e., cathode on the inlet side). A μSIL-DB-1 coated capillary (catalog No. 197-1002, J&W Scientific, Folsom, CA, USA) with a 100 μm I.D., 0.1 μm film thickness, and length of 27 cm (20 cm to the detector) was housed in a P/ACE cartridge (Beckman Coulter) and maintained at 25°C. The sieving buffer consisted of 0.5% (w/v) HPMC in 1X TBE (89 m*M* Tris–base, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.3). Immediately prior to use, an appropriate amount of intercalating dye: 10 μl of EnhanCE (1 mg/ml stock in methanol), 10 μl of YO-PRO-1 (1 m*M* stock in dimethyl sulfoxide, DMSO) or 2 μl of SYBR Green I (10 000×concentrate in DMSO) was added to 20 ml of sieving buffer, which was mixed for 15–30 min and degassed by sonnication for 1 min. The sample injection/run routine was as follows: (1) prior to the run the capillary was rinsed at high pressure (20 p.s.i.; 1 p.s.i.=6894.76 Pa) for 1 min with sieving buffer (not subjected to prior electrophoresis); (2) diluted (1:100 in deionized water) RT-PCR samples (100 or 200 μl total volume) were subjected to electrokinetic loading at 3.0 kV for 5–10 s; (3) separations were performed at a field strength of 300 V/cm (8.1 kV) for 6 min; (4) at the end of each run, the capillary was rinsed at high pressure (20 p.s.i.) with sieving buffer for an additional 1 min. At the beginning and end of each day of use, the capillary was regenerated by rinsing consecutively with water, methanol, and water again for 5 min each. The *Hae*III digest of ΦX174 DNA, which contains 11 different restriction fragments ranging in size from 72 bp to 1353 bp, was used to evaluate capillary and instrument performance prior to running actual samples (see Fig. 3 for a typical separation).

2.6. Data analysis

Peak areas were calculated by P/ACE Station software (Beckman Coulter). To compensate for differences in the amount of dye binding to DNA fragments, which is dependent on fragment length

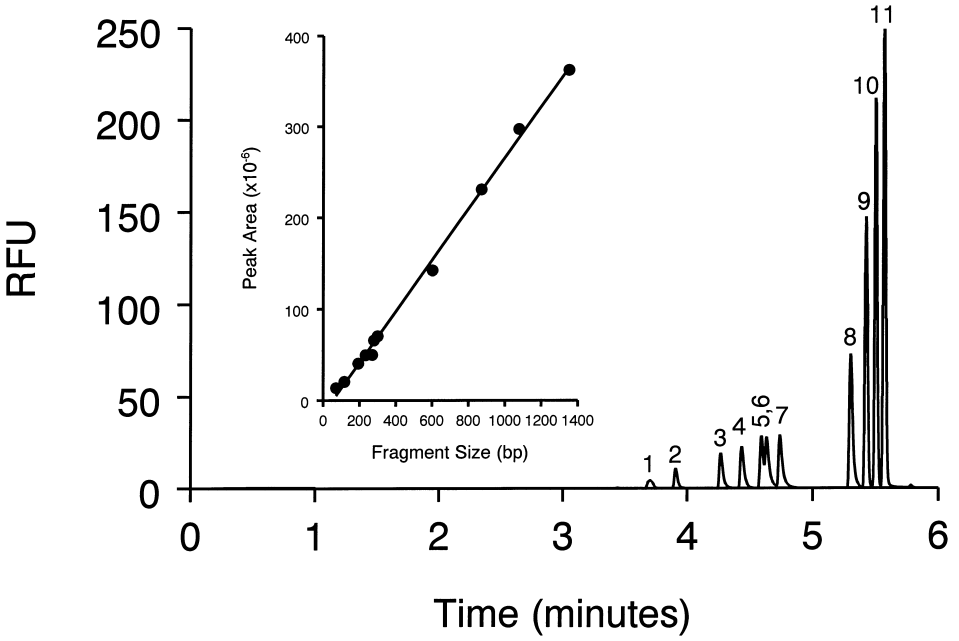

Fig. 3. CE–LIF separation of a sample (200 ng/ml) of a *Hae*III digest of ΦX174 DNA containing 11 restriction fragments ranging in size from 72 bp (peak 1) to 1353 bp (peak 11). The insert depicts a linear regression plot of the integrated peak area vs. size (in bp) of each fragment.

(Fig. 3, insert), the peak area for the β-actin peak was multiplied by a factor (261/612) that accounted for the size difference (in bp) between the leptin and β-actin amplicons. A corrected leptin/β-actin peak area ratio was then calculated and used to compare individual samples. Ratio values are expressed as mean±SD and mean comparisons between experimental treatments were made using a two-tailed, unpaired Student's *t*-test with the level of statistical significance set at $P \leq 0.05$.

For QC-RT-PCR, the log of the ratio of the integrated peak areas for leptin competitor and target amplicons was calculated. To compensate for differences in the amount of dye bound, the area of the competitor peak was multiplied by a factor (261/234) to reflect the size difference (in bp) between target and competitor amplicons. Linear regression analysis of the log of the ratio of corrected competitor/target integrated peak areas vs. the log of the amount (amol) of competitor added to each reaction mixture generated an equation that was used to calculate the amount (amol) of of leptin mRNA (where log competitor/target=0) present in the total RNA sample (5 μg) subjected to RT-PCR.

3. Results and discussion

3.1. CE–LIF technique

The CE–LIF technique developed in this study to analyze leptin gene expression is based on previously published work [16–23]. We used EnhanCE as the intercalating dye and, in conjunction with a DB-1 coated capillary and HPMC-containing sieving buffer matrix, we were able to separate 11 dsDNA restriction fragments ranging in size from 72 to 1353 bp in under 6 min (Fig. 3). This separation provides sufficient coverage for the size range of dsDNA amplicons (100–800 bp) typically produced in most RT-PCR reactions [19]. Two fragments, differing in size by 10 bp (see Fig. 3, peaks 5 and 6), were not completely resolved. Similar resolution, but shorter overall separation time, has been reported for ΦX174

dsDNA restriction fragments when ethidium bromide was used as the intercalating dye in an acrylamide-based gel buffer with a shorter (12 cm) DB-1 capillary [24]. Thus, the lower limit of fragment size resolution for our separation technique appears to be around 10 bp. A strict linear relationship was observed between the amount of EnhanCE bound (as measured by integrated peak area) and the size (in bp) of the dsDNA fragment (Fig. 3, insert).

Fig. 4 depicts the separation of an RT-PCR mixture containing chicken liver total RNA, which produced amplicons for both leptin and β-actin. Individual aliquots of this RT-PCR sample were separated in the presence of three different fluorescent intercalating dyes to evaluate their utility in CE–LIF. Both YO-PRO-1 and SYBR Green I, when added to the HPMC sieving buffer, resulted in increased migration times, increased peak resolution, and increased quantum fluorescence yield for both amplicons and for the individual dsDNA restriction fragments of ΦX174 (data not shown) compared to EnhanCE. Similar findings were reported previously for the comparison of SYBR Green I, YO-PRO-1, and thiazole orange used in the analysis of dsDNA restriction fragments and PCR products [25]. The slower migration (increased migration time) results from a decrease in the net negative charge of the dsDNA–dye complex as compared to dsDNA without intercalating dye bound [26]. SYBR Green I dye produced a specific anomalous effect on the peak shape of the leptin amplicon. The fronting shoulders (see arrows Fig. 4) and peak broadening indicate polydispersity in the leptin dsDNA fragment possibly resulting from the binding of differing numbers of dye molecules or the binding of dye molecules to different sites [27]. This behavior was not observed for the β-actin dsDNA fragment, which does differ from the leptin amplicon not only in size, but also in its GC content. Because of these observations, we chose to use the EnhanCE intercalating dye in developing our CE–LIF analysis of RT-PCR samples to provide optimum peak shape and separation time.

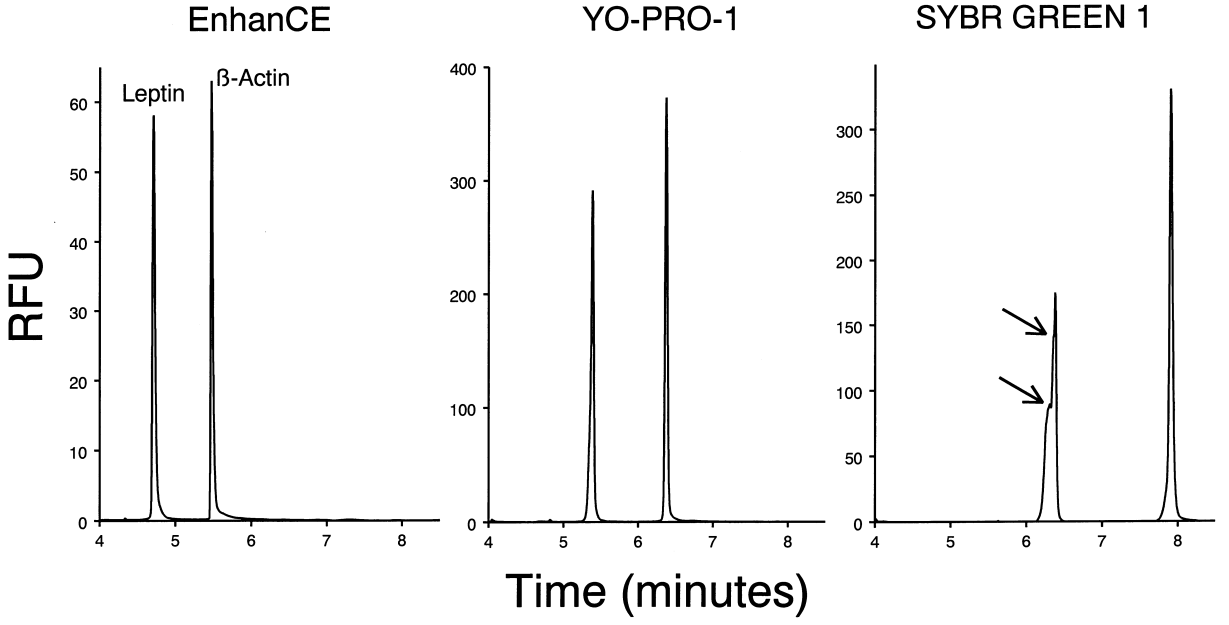

Fig. 4. CE–LIF of an RT-PCR sample containing 5 μg total RNA isolated from an 8-week-old male broiler chicken liver separated in the presence of three different fluorescent intercalating dyes. The separation buffer consisted of 0.5% HPMC in 1X TBE buffer with 10 μl of EnhanCE, 10 μl of YO-PRO-1, or 2 μl of SYBR Green I added per 20 ml of separation buffer. All other separation conditions were held constant except that the total run time was extended from 6 to 9 min to accommodate the effects of the different intercalating dyes on peak migration times. The leptin and β-actin amplicons are each labeled.

Although, SYBR Green I and YO-PRO-1, as well as the dimeric, asymmetric cyanine dyes, such as TOTO and YOYO, have a very high binding affinity for dsDNA and produce a marked enhancement in the quantum yield of fluorescence [25,28], the detection sensitivity achieved using EnhanCE was found to be sufficient for the analysis of typical RT-PCR samples.

3.2. Leptin RT-PCR

In the interest of simplifying the analysis of RT-PCR samples we sought to avoid extensive manipulation of samples, such as centrifugal ultrafiltration and membrane desalting, as employed by others to counteract the negative effects of buffer constituents on sample loading by electrokinetic injection. Substantial losses of dsDNA have been reported to occur during centrifugal ultrafiltration [19]. Instead, we chose to simply dilute the reactions and load them directly. Table 1 summarizes data collected to evaluate the effects of dilution and sequential injection from the same RT-PCR sample (200 μl volume) containing chicken liver total RNA on the integrated areas of the leptin and β-actin amplicon peaks and on their corrected ratio. From this data, it is clear that the extent of dilution has an impact on the amount of sample loaded, as judged by the integrated peak area values for both leptin and β-actin amplicons. Moreover, increasing the dilution led to a small increase in peak area ratios obtained from the initial injection of each sample. It is also clear that further injections

Table 1
Effects of dilution and consecutive electrokinetic injections on RT-PCR sample analysis by CE–LIF

Dilution[a]	Injection number	Leptin (L) peak area	β-Actin (A)[b] peak area	L/A ratio	Average	SD	RSD (%)
1:20	1	70 452 056	53 188 436	1.32	1.28	0.05	4.06
	2	82 642 968	61 132 962	1.35			
	3	109 793 648	86 839 258	1.26			
	4	124 291 200	100 123 312	1.24			
	5	117 809 888	95 405 183	1.23			
1:50	1	69 579 488	54 004 339	1.29	1.30	0.11	8.62
	2	107 950 696	81 266 195	1.33			
	3	155 716 000	130 195 343	1.20			
	4	164 605 136	135 885 703	1.21			
	5	69 837 608	47 344 701	1.48			
1:100	1	90 298 160	67 173 184	1.34	1.32	0.115	11.77
	2	198 925 088	148 346 477	1.34			
	3	137 064 672	120 632 904	1.14			
	4	129 112 064	106 141 283	1.22			
	5	28 074 804	18 172 570	1.54			
1:200	1	105 937 568	73 344 814	1.44	1.24	0.14	11.45
	2	226 065 280	177 746 213	1.27			
	3	504 170 080	419 432 309	1.20			
	4	579 814 528	464 681 207	1.25			
	5	269 536 960	257 116 757	1.05			
1:400	1	9 156 918	6 355 356	1.44	1.35	0.11	8.24
	2	39 899 668	27 251 307	1.46			
	3	52 904 744	38 444 413	1.38			
	4	77 539 040	61 567 239	1.26			
	5	44 752 036	36 956 976	1.21			

[a] Sample volumes were 200 μl in all cases and each sample was loaded into the capillary by electrokinetic injection for 10 s.
[b] Corrected peak area.

beyond the initial one result in progressively increasing amounts of sample being loaded until the point of analyte depletion is reached (for most samples by the fifth injection). This is most likely due to the removal of substances (e.g., ions) impeding the electrokinetic loading of the dsDNA amplicons. Repeated electrokinetic injections from the same sample also significantly affected the leptin/β-actin ratio such that the RSDs for the average of five injections exceeded 5% in all but the 1:20 dilution group. Since the ratios changed upon repeated injections, a variable loading efficiency for the β-actin amplicon relative to that of leptin may exist under these conditions. Guttman and Schwartz [29] observed progressively smaller peak heights resulting from consecutive electrokinetic injections of DNA from the same low volume (10–200 μl) of aqueous sample. A comparison of the average of the leptin/β-actin ratio for the first injection of each dilution listed in Table 1 (1.37±0.07, RSD=5.16%) compares quite favorably with the average of five individually prepared samples, each diluted 1:100 (1.34±0.07, RSD=5.42%). This result indicates that sample preparation is probably the chief source of variability and that there is some degree of flexibility in the level of dilution for an effective CE–LIF analysis. In all subsequent work we chose to routinely dilute RT-PCR samples 1:100 and subject them to a single electrokinetic injection.

Fig. 5 depicts an evaluation of the number of PCR cycles relative to the amount of leptin and β-actin amplicons produced (as judged by integrated peak areas). It is clear that 30 cycles is contained within the exponential phase (linear portion of the log–linear plot in Fig. 5) of PCR for both amplicons. Furthermore, although the β-actin amplicon is produced in higher amounts than leptin in all samples, the kinetics of PCR appear to be quite similar for both amplicons. Based on these findings, 30 cycles was chosen for use in all subsequent PCR reactions. This number of PCR thermal cycles has previously been determined to be optimal for both leptin and β-actin amplicons analyzed by agarose slab gel electrophoresis with ethidium bromide staining [11].

Total RNA was isolated from a variety of tissues and analyzed for the presence of leptin mRNA. Fig. 6 compares typical RT-PCR results obtained for total RNA samples isolated from liver, adipose, and skeletal muscle tissue. It is clear that in chickens leptin is expressed only in liver and adipose tissue

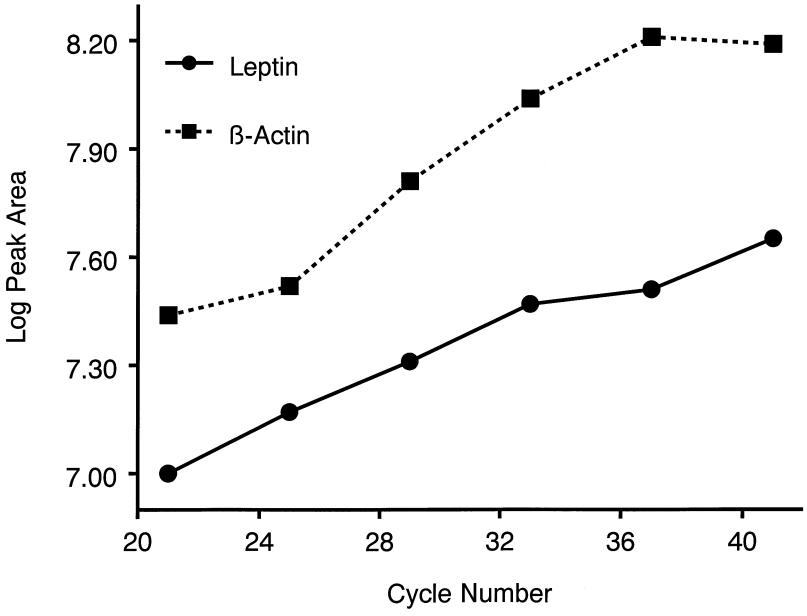

Fig. 5. Plots of the log of the integrated peak areas of leptin and β-actin amplicons as a function of the number of PCR thermal cycles. The RT-PCR mixture contained 5 μg total RNA isolated from the liver of an 8-week-old male broiler chicken.

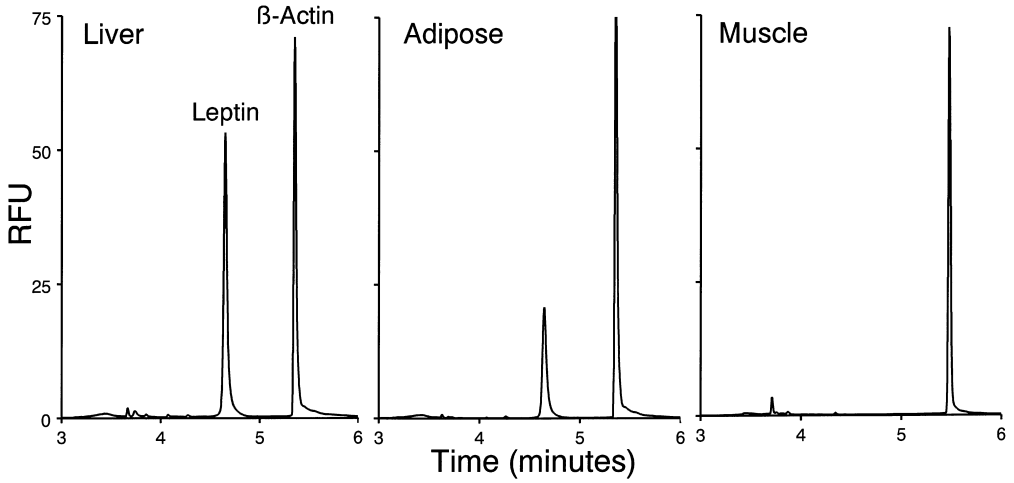

Fig. 6. CE–LIF separation of RT-PCR samples containing 5 μg total RNA isolated from liver, adipose (abdominal fat pad), or skeletal muscle tissue collected from 8-week-old male broiler chickens. The leptin and β-actin amplicons are each labeled.

with no detectable expression in skeletal muscle or any other tissue studied so far [11]. The high level of hepatic leptin expression is consistent with previous findings and indicative of the important role that the avian liver plays in the regulation of lipogenesis and energy metabolism [12]. Moreover, unlike mammals where leptin is primarily expressed in white adipose tissue it appears that in birds the primary site of leptin expression is liver and to a lesser extent adipose tissue [10,11]. This fact was confirmed by a comparison of the ratio of the integrated peak area of the leptin amplicon to that of β-actin. This ratio has been used previously to quantify avian leptin gene expression by RT-PCR and agarose slab gel electrophoresis with ethidium bromide staining [11]. Similarly, a recent report demonstrated the utility of a coamplified internal standard (cyclophillin) for relative RT-PCR quantitation of sodium channel subunit mRNAs using multiplexed CE–LIF with primer dropping [22]. Therefore, in our initial studies, we have chosen to use the leptin/β-actin ratio as a quick and convenient method for comparing leptin expression among different tissue RNA samples.

3.3. Effects of estrogen and insulin treatment on leptin gene expression

Fig. 7 shows a comparison of leptin expression in liver and adipose (abdominal fat pad) tissue samples from 4-week-old male broiler chickens treated with estrogen. Estrogen treatment resulted in a decrease in leptin expression in both liver and adipose tissue of approximately 1.5-fold. This decline was significant ($P<0.05$) for liver. Conversely, insulin treatment of 12-week-old male broiler chickens resulted in increases of leptin expression in liver and adipose tissue of 2.2- and 1.4-fold, respectively (Fig. 8). Again, only the increase in liver was determined to be significant ($P<0.05$). There is substantial evidence in mammals to indicate that insulin regulates leptin expression in white adipose tissue and that this hormone appears to be a primary factor [6]. The results of this study agree with a previous report of the effects of insulin and estrogen on leptin expression in chickens from our laboratory [11] and further indicate that the liver appears to be more responsive to hormonal regulation of leptin expression than does adipose tissue. In fact, it has been suggested that in chickens basal expression of leptin in adipose tissue is already at or near maximal levels or the mechanism governing its expression is different from that in liver [11]. Either of these two scenarios could account for the differential level of leptin expression in liver vs. adipose tissue observed in this study.

Comparing the leptin/β-actin ratios for both liver and adipose tissue from the different aged (4 or 12-week-old) birds used in this study suggested an increase in leptin gene expression with age (compare

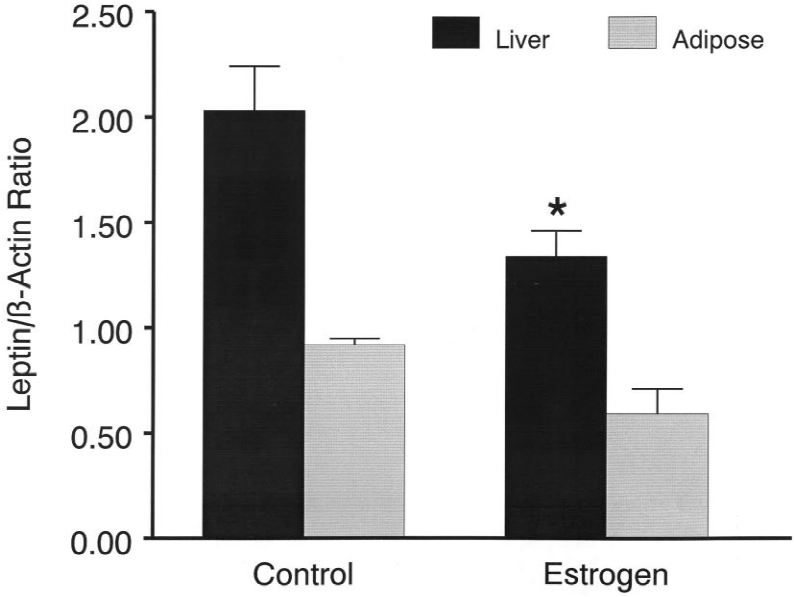

Fig. 7. The effects of estrogen (20 mg of 17β-estradiol injected im) on leptin expression in liver and adipose (abdominal fat pad) tissue obtained from 4-week-old male broiler chickens. The ratio of leptin to β-actin amplicons (corrected for size difference) was used to quantify the relative level of leptin expression. The asterisk indicates a statistically significant difference ($P<0.05$) for the mean comparison of treated vs. control samples.

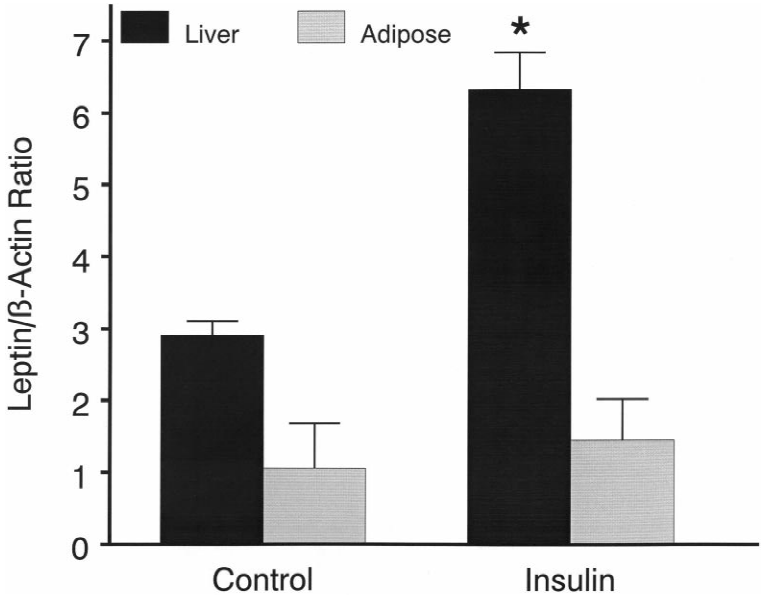

Fig. 8. The effects of insulin (200 μg porcine insulin/kg body mass/day im for a period of four days) on leptin expression in liver and adipose (abdominal fat pad) tissue from 12-week-old male broiler chickens. The ratio of leptin to β-actin amplicons (corrected for size difference) was used to quantify the relative level of leptin expression. The asterisk indicates a statistically significant difference ($P<0.05$) for the mean comparison of treated vs. control samples.

liver and adipose tissue control values in Figs. 7 and 8). The effect of age on leptin expression in liver and adipose tissue is currently being investigated by our laboratory. We have also applied RT-PCR–CE–LIF to the analysis of leptin expression in liver and yolk sac tissues taken from chick embryos at different stages of their development [30]. Thus, RT-PCR–CE–LIF offers a rapid means for assessing relative changes in leptin gene expression in response to experimental treatments, stage of development, or changes in physiological state.

3.4. Quantitative-competitive RT-PCR assay for leptin gene expression

A number of investigators have pointed out the potential pitfalls of generating non-reproducible or inaccurate estimates of mRNA levels by the use of relative or semi-quantitative RT-PCR analysis methods [19,23]. This criticism is applicable to both conventional agarose slab gel electrophoretic and CE–LIF analyses. Although, QC-RT-PCR methods are difficult to develop and conduct, they do offer one of the most accurate ways to directly quantify mRNA levels. In order to achieve more accurate estimates of leptin gene expression, we have constructed a specific chicken leptin internal competitor that produces a 234 bp amplicon that is 27 bp smaller than the target amplicon (261 bp) and cloned it into an expression vector for the production of synthetic RNA (see Fig. 2). The same gene-specific primer set can be utilized to generate both the

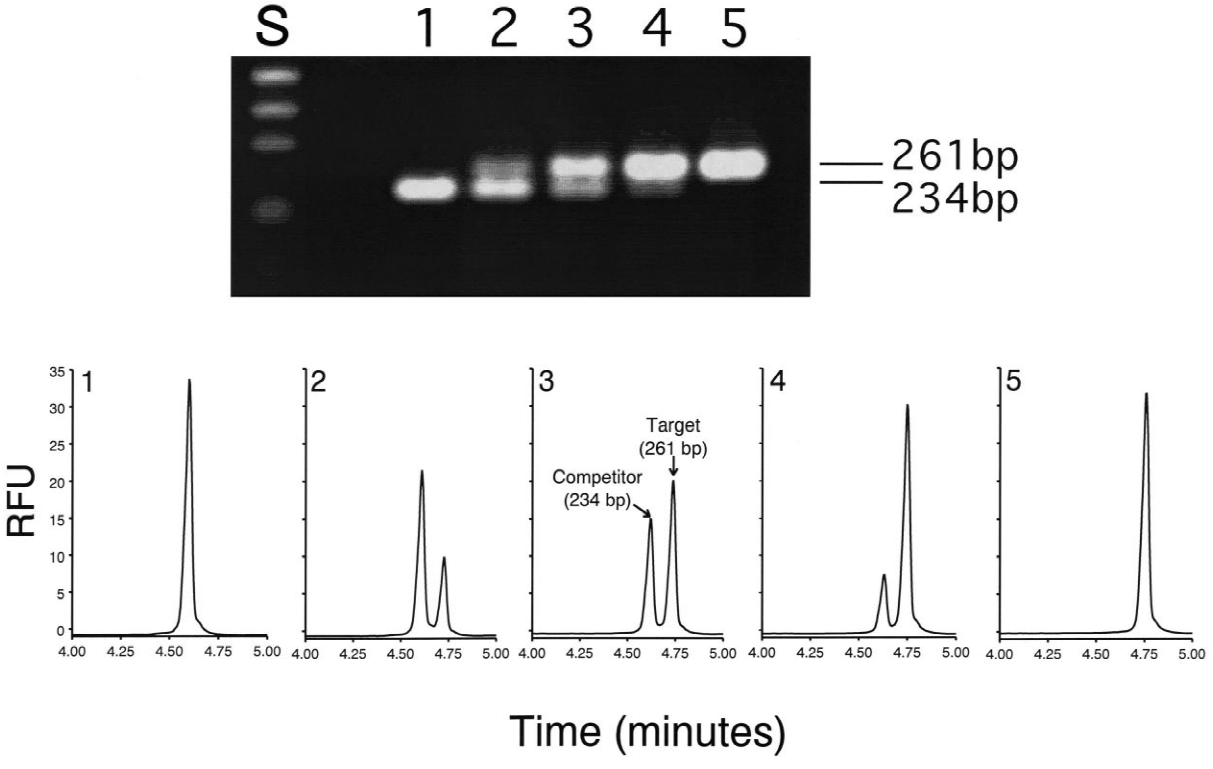

Fig. 9. A comparison of agarose slab gel electrophoresis with ethidium bromide staining (top) and CE–LIF separations of dsDNA chicken leptin amplicons (target=261 bp; competitor=234 bp) produced by RT-PCR amplification of synthetic RNAs (250 amol/μl) transcribed from the full length coding sequence (target) and the 27 bp deletion construct (internal competitor) plasmids (see Fig. 2 for details). Depicted in the agarose slab gel separation are: Lanes: 1=5 μl competitor RNA; 2=3.8 μl competitor and 1.2 μl full length RNAs; 3=2.5 μl each of the competitor and full length RNAs; 4=1.2 μl competitor and 3.8 μl full length RNAs; 5=5 μl full length RNA. Molecular mass standards are shown at the top left (lane S). Below are the corresponding samples (lanes 1–5) separated by CE–LIF. A 1 min section (4–5 min) of the CE–LIF electropherogram for each sample is shown with the numbers corresponding to the lane (sample) numbers depicted in the agarose slab gel separation shown above.

chicken leptin target and competitor amplicons. Fig. 9 depicts a series of control reactions containing differing amounts of full length and competitor synthetic RNAs subjected to RT-PCR and analyzed by agarose slab gel (2%) electrophoresis with ethidium bromide staining and by CE–LIF for comparison. The separations depicted indicate that the CE–LIF separation method is more than adequate for the analysis of typical RT-PCR samples in a quantitative-competitive mode.

Because QC-RT-PCR offers the potential for a more precise estimate of leptin mRNA, this type of analysis may be particularly suited to assess subtle changes in leptin expression, such as those most likely to occur in adipose tissue. To demonstrate this samples of liver and adipose tissue total RNA (5 μg) obtained from 8-week-old male broiler chickens were subjected to RT-PCR in the presence of five different levels of competitor RNA. Aliquots of the reactions were then subjected to analysis by agarose slab gel (2%) electrophoresis and CE–LIF (Fig. 10). Using linear regression analysis of CE–LIF derived

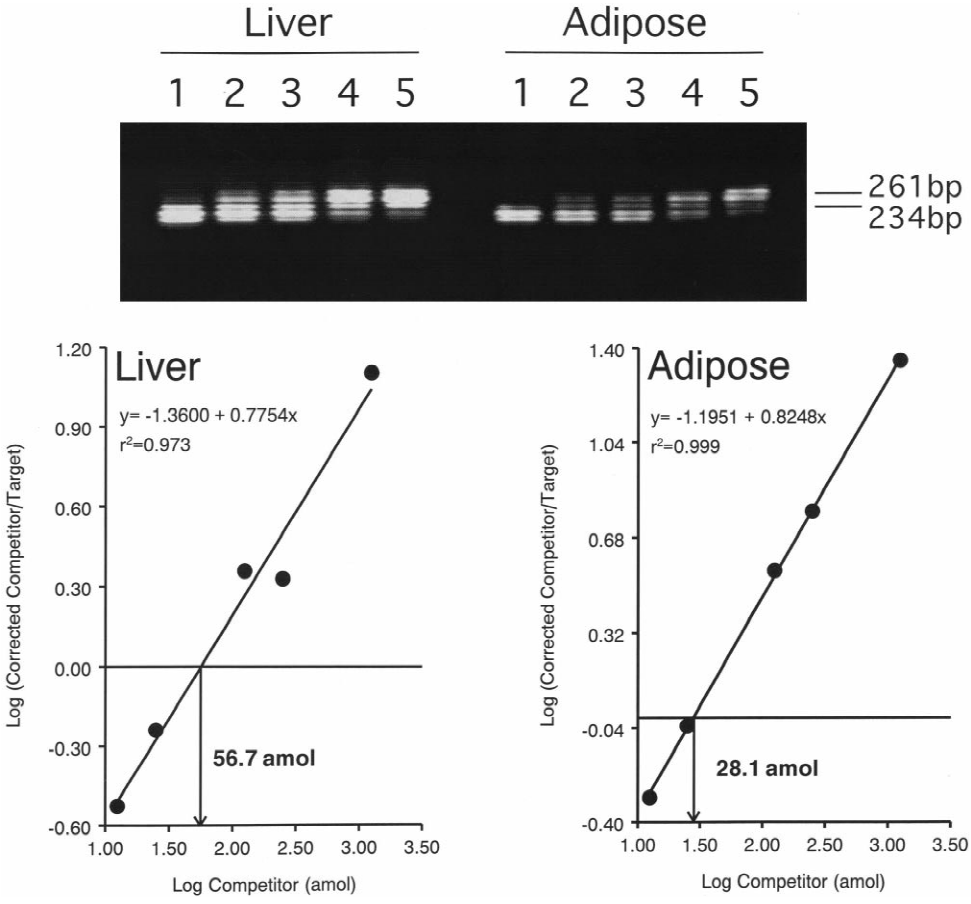

Fig. 10. QC-RT-PCR analysis of total RNA (5 μg) isolated from liver and adipose tissue samples obtained from 8-week-old male broiler chickens. In addition to the total RNA, reactions also contained 1250, 250, 125, 25, or 12.5 amol of competitor synthetic RNA (lanes 1–5, respectively) and a portion of the each reaction was separated by agarose slab gel electrophoresis with ethidium bromide staining (top). A portion (2 μl) of each sample was also diluted (1:100) and subjected to CE–LIF separation. CE–LIF was sufficiently sensitive to detect small amounts of target amplicons present in lane 1 (both liver and adipose tissue samples) that were not apparent in the ethidium bromide stained agarose slab gels. The integrated peak areas were determined for the ratio of competitor and target amplicons (corrected for the size difference between competitor and target). The log of this ratio was plotted against the log of the added amount of competitor RNA and linear regression analysis was used to calculate the amount of leptin mRNA (in amol) contained in the original RNA sample (5 μg) subjected to RT-PCR as indicated on each plot.

data, a plot of the log of corrected competitor/target integrated peak areas vs. the log of competitor added to the reaction mixture (amol) was used calculate the amount of leptin mRNA contained in the 5 μg sample of total RNA analyzed. The results indicated that leptin expression in liver is twice that of adipose tissue (56.7 vs. 28.1 amol), thus confirming a higher basal level of leptin expression in liver vs. adipose tissue. This is also remarkably similar to a comparison of liver and adipose tissue leptin/β-actin ratios determined from the relative quantitative RT-PCR technique discussed previously (2.21 at 4 weeks and 2.74 at 12 weeks; for control values in Figs. 7 and 8).

4. Conclusions

This study is the first to report on the application of CE–LIF to the analysis of leptin gene expression as measured by RT-PCR. We have developed a rapid (<6 min) separation method involving CE–LIF to detect and quantify amplicons for leptin and β-actin amplified from chicken liver and adipose tissue total RNA samples. By determining the level of leptin amplicon relative to that of β-actin, we were able to assess the effects of hormonal treatment on leptin gene expression in the liver and adipose tissue from male broiler chickens. We have also developed and evaluated the necessary reagents (internal competitor for chicken leptin) for developing a QC-RT-PCR assay for leptin expression with CE–LIF detection and quantification of the amplified sequences. Since no techniques currently exist for the measurement of chicken leptin protein, QC-RT-PCR–CE–LIF represents a promising method by which to accurately assess leptin gene expression in chickens. This is especially true for adipose tissue where incremental changes in leptin expression are expected to be small as compared to liver.

Acknowledgements

The authors wish to acknowledge Dr. James M. Hempe (LSU, School of Medicine, Department of Pediatrics, New Orleans, LA, USA) for his helpful advice and many practical suggestions which were instrumental in the development of the CE–LIF technique used in this study. The authors also wish to acknowledge contributions made by Dr. Susan M. Czerwinski during the initial stages of this work and Dr. Melissa S. Ashwell for her assistance with DNA sequencing. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the US Department of Agriculture and does not imply its approval to the exclusion of other suitable products.

References

[1] Y. Zhang, R. Proenca, M. Maffei, M. Barone, L. Leopold, J.M. Friedman, Nature 372 (1994) 425.
[2] M.A. Pellymounter, M.J. Cullen, M.B. Baker, R. Hecht, D. Winters, T. Boone, F. Collins, Science 269 (1995) 540.
[3] J.L. Halaas, K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Rabinowitz, R.L. Lallone, S.K. Burley, J.M. Friedman, Science 269 (1995) 543.
[4] F.E. Chehab, M.E. Lim, R.H. Lu, Nat. Genet. 12 (1996) 318.
[5] A.D. Kline, G.W. Becker, L.M. Churgay, B.E. Landen, D.K. Martin, W.L. Muth, R. Ratnachalam, J.M. Richardson, B. Schoner, M. Ulmer, J.E. Hale, FEBS Lett. 407 (1997) 239.
[6] J.M. Friedman, J.L. Halaas, Nature 395 (1998) 763.
[7] L.A. Tartaglia, M. Dembski, X. Weng, N.H. Deng, J.J. Culpepper, R. Devos, G.J. Richards, L.A. Campfield, F.T. Clark, J. Deeds, C. Muir, S. Sanker, A. Moriarity, K.J. Moore, J.S. Smutko, G.G. Mays, E.A. Woolf, C.A. Monroe, R.I. Tepper, Cell 83 (1995) 1263.
[8] L.A. Campfield, F.J. Smith, Y. Guisez, R. Devos, P. Burn, Science 269 (1995) 546.
[9] K.L. Houseknecht, C.P. Portocarrero, Domest. Anim. Endocrinol. 15 (1998) 457.
[10] M. Taouis, J.-W. Chen, C. Daviaud, J. Dupont, M. Derouet, J. Simon, Gene 208 (1998) 239.
[11] C.M. Ashwell, S.M. Czerwinski, D.M. Brocht, J.P. McMurtry, Am. J. Physiol. 276 (1999) R226.
[12] J. Simon, B. Chevalier, M. Derouet, B. LeClercq, J. Nutr. 121 (1991) 379.
[13] C.I. Rosenblum, A. Vongs, M.R. Tota, J.P. Varnerin, E. Frazier, D.F. Cully, M.A. Morsy, L.H.T. Van der Ploeg, Mol. Cell. Endo. 143 (1998) 117.
[14] J. Rentsch, M. Chiesi, FEBS Lett. 379 (1996) 55.
[15] D. Auboeuf, H. Vidal, Anal. Biochem. 245 (1997) 141.
[16] E.F. Rossomando, L. White, K.J. Ulfelder, J. Chromatogr. B 656 (1994) 159.
[17] J.M. Butler, B.R. McCord, J.M. Jung, M.R. Wilson, B. Budowle, R.O. Allen, J. Chromatogr. B 658 (1994) 271.
[18] A.W.H.M. Kuypers, J.P.P. Meijerink, T.F.C.M. Smetsers, P.C.M. Linssen, E.J.B.M. Mensink, J. Chromatogr. B 660 (1994) 271.

[19] M.J. Fasco, C.P. Treanor, S. Spivack, H.L. Figge, L.S. Kaminsky, Anal. Biochem. 224 (1995) 140.
[20] U. Vincent, G. Patra, J. Therasse, P. Gareil, Electrophoresis 17 (1996) 512.
[21] D. Personett, K. Sugaya, D. Hammond, M. Robbins, M. McKinney, Electrophoresis 18 (1997) 1750.
[22] V.M. Vehaskari, J.M. Hempe, J. Manning, D.H. Aviles, M.C. Carmichael, Am. J. Physiol. 274 (1998) C1661.
[23] N.D. Borson, M.A. Strausbauch, P.J. Wettstein, R.P. Oda, S.L. Johnston, J.P. Landers, Biotechniques 25 (1998) 130.
[24] C. Barta, M. Sasvari-Szekely, A. Guttman, J. Chromatogr. A 817 (1998) 281.
[25] J. Skeidsvoll, P.M. Ueland, Anal. Biochem. 231 (1995) 359.
[26] H.E. Schwartz, K.J. Ulfelder, Anal. Chem. 64 (1992) 1737.
[27] D.H. Atha, Electrophoresis 19 (1998) 1428.
[28] K. Srinivasan, S.C. Morris, J.E. Girard, M.C. Kline, D.J. Reeder, Appl. Theoret. Electrophoresis 3 (1993) 235.
[29] A. Guttman, H.E. Schwartz, Anal. Chem. 67 (1995) 2279.
[30] C.M. Ashwell, M.P. Richards, D.M. Brocht, J.P. McMurtry, FASEB J. 13 (1999) A442.